Modulation of human neutrophil apoptosis by gut hormones

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Abstract

Background: Inhibition of neutrophil apoptosis seems to be a prominent feature in inflammation, parenchymal damage, and unresolved organ dysfunction. Agents that modulate neutrophil apoptosis could alter the course of the disease. A variety of endogenous peptides, including some GIT hormones, have been reported as modulators of apoptosis in several types of cells.

Objectives: We aim to study the effect of the GIT hormones VIP, gastrin, secretin, GIP and CCK on the *in vitro* viability of human neutrophils and the associated caspase changes.

Methods: Neutrophils were prepared from the venous blood of twenty healthy male volunteers using density gradient centrifugation. Neutrophils were cultured in RPMI and treated with two concentrations of each of the five hormones and incubated for 1, 2, 4 and 6 hours. Viability was assessed by a kit that measured ATP levels. Caspase 3/7, caspase 8 and caspase 9 were measured using specific kits.

Results: VIP accelerated apoptosis causing a 15 – 32% decrease in neutrophil viability in 11 of the twenty volunteers. VIP increased the levels of caspase 8 and caspase 3/7. Gastrin suppressed apoptosis causing a 17-30% increase in neutrophil viability in 7 of the twenty volunteers. However, VIP inhibited apoptosis in one subject and gastrin accelerated apoptosis in two subjects. The effects of secretin, GIP and CCK were not marked.

Conclusions: The modulatory effects of VIP and gastrin on human neutrophil apoptosis are seen in a proportion, not all, of the population. VIP is more consistent in its effects than gastrin.

Key Words: VIP, Gastrin, Neutrophil, Apoptosis, Caspase3/7, Caspase 8.

Introduction

Neutrophils are an essential component of innate immunity. They are produced continuously at a high rate and the same number of cells should, therefore, die at the same rate in order to keep cellular homeostasis under physiologic conditions. Changing the rate of apoptosis produces rapid changes in neutrophil numbers.[1] It has been reported that inhibition of neutrophil apoptosis is a prominent feature of autoimmune inflammation, parenchymal damage, and unresolved organ dysfunction.[2]

A variety of endogenous peptides, including some gastrointestinal (GIT) hormones, are thought to modulate apoptosis of several cell types, like respiratory epithelial cells, endothelial cells, fibroblasts and lymphocytes.[3,4] In those studies, the vasoactive intestinal peptide (VIP) was the GIT hormone that appeared most promising to be a modulator of apoptosis of various cell types.

The physiological effects of various GIT hormones appear to extend beyond the mere regulation of the secretions and motility of the gut.[5, 6] We and other researchers have previously described that VIP, gastrin and other gut hormones are potential modulators of the adaptive immune response in terms of cytokine production by lymphocytes and enhancement of the migratory and phagocytic activity of macrophages.[7-9]

In this study we explored the possibility that some of the GIT hormones are potential modulators of neutrophil apoptosis. The five gut hormones VIP, gastrin, secretin, gastric inhibitory peptide (GIP) and cholecystokinin (CCK), were studied for their ability to alter, *in vitro*, the viability of freshly prepared human neutrophil granulocytes over a period of several hours. If a change in neutrophil viability was observed, the associated changes in initiator and executor caspase levels were analyzed.

Materials and Methods

The cell culture medium Rosewell Park Memorial Institute – 1640 (RPMI-1640) supplemented with HEPES, the antibiotic solutions, phosphate buffered saline, human recombinant VIP, gastrin, secretin, GIP and CCK, in addition to histopaque density gradient solutions (1.077 and 1.119) were supplied by Sigma Aldrich Company. The kits used for measuring cell viability (CellTiter-Glo), caspase 3/7 (Caspase-Glo 3/7), caspase 8 (Caspase-Glo 8) and caspase 9 (Caspase-Glo 9) were supplied by Promega Corporation. The black-walled cell culture plates with 96 clear flat-bottomed wells were from Corning.

Preparation of the culture medium

Serum-free RPMI medium was prepared by adding L-Glutamine (0.3 g/L) and antibiotics to a final concentration of 100 u/ml penicillin and 100 µg/ml streptomycin.

Preparation of granulocytes

Twenty healthy male volunteers from preclinical classes of Qassim College of Medicine donated the blood samples. Ten ml of peripheral venous blood samples were collected in heparinized tubes (final concentration of heparin was 20 units/ml of blood), and diluted 1:1 with phosphate buffered saline. The granulocytes were prepared using density gradient centrifugation based on the method developed by Boyum.[10] Histopaque 1.119 was overlayed by histopaque 1.077 and the diluted blood was added on top and centrifuged as previously described.[11] Granulocytes were harvested from the interface between the two histopaque solutions. The granulocytes were then washed, exposed to a cycle of hypotonic hemolysis, resuspended in RPMI and counted in a haemocytometer. Neutrophils constituted about 95% of the cells.

Each volunteer donated two blood samples on two different occasions. The neutrophils of the first sample were tested for the effects of the GIT hormones on cell viability. The neutrophils of the second sample were tested for the effects of the GIT hormones on caspase levels.

GIT hormones

Two final concentrations were used in our cultures for each of the GIT hormones, VIP, gastrin, secretin, GIP and CCK. The concentrations were: 1 ng/ml and 5 ng/ml. The hormones were added at the start of the cultures. The stock solutions of the hormones were diluted to the working concentrations in serum-free RPMI.

Culture of peripheral blood neutrophils

Neutrophils were cultured immediately after separation and counting. The time from blood collection to start of cultures was about 3 hours. Cells were cultured in black-walled 96-well plates with clear flat bottom wells. Both control and the test cultures were done in triplicates. The number of cells per well was 30,000 for the viability tests and for the assays of caspase 8 and caspase 9. The number of cells per well was 20,000 for the caspase 3/7 assay. In each plate there were two sets of cultures for each of the 5 GIT hormones; one to be treated with 1 ng/ml and one to be treated with 5 ng/ml final concentration of the hormone. Serum-free RPMI was added to the control cultures instead of the hormone solution. The final volume per well was 110 µl.

For each volunteer 4 plates were used so as to do the measurements at 1 hour, 2 hours, 4 hours and 6 hours. Plates were incubated at 37 ^{0}C in 5% CO_2.

Measurement of viability and caspase levels

The test of viability is based on measuring the amount of ATP which corresponds to the number of viable cells. The tests for the caspases specifically measured the activity of each of caspase 3/7, caspase 8 and caspase 9. All tests were done by adding to each well a volume of the specific reagent equal to the culture volume, shaken and let to stand according to Promega protocols and the luminescence was then measured.[12] The plate reader used was Mithras LB940 from Berthold Technologies using MikroWin 2000 software.

Statistical methods

Results of all tests were analyzed by using SPSS statistical analysis program. For each volunteer, the mean was calculated for each triplicate wells of controls and tests. The difference between the mean value of hormone-treated cultures and the mean value of their respective control was calculated and expressed as a percent of the control to give the percent change according to the formula:

Hormone induced percent change =

$$\frac{\text{Mean of Test Wells - Mean of Control wells}}{\text{Mean of Control wells}} \times 100$$

For each hormone, the percent changes in viability from all twenty samples were arranged in ascending order to determine the median percent change and range of percent changes induced by the hormone. Paired samples t-test was used for significance testing.

Results

Measurements of neutrophil viability were done after 1 hour, 2 hours, 4 hours and 6 hours of incubation. The neutrophils of the different subjects showed their maximum response to the GIT hormones at different points in time. The largest hormone-induced change in viability (relative to control) was detected at 4 hours for some volunteers and at 6 hours for others. Viability changes were less marked at two hours and not detectable at 1 hour. For each volunteer, the largest hormone-induced viability change (out of the four measurements) was taken as the maximum response of that volunteer to that hormone and was used for comparison with others.

Responders and non-responders

When the percent changes in neutrophil viability for all twenty volunteers were arranged in ascending order (each hormone separately), it was immediately obvious that there was a cluster of non-responders to the GIT hormones in whom the percent change in viability between test and control was less than 5%. The rest of the subjects were responders with percent changes of more than 14%.

Table 1 shows that VIP suppressed the viability of neutrophils in 11 of the 20 volunteers and enhanced it in one volunteer. The VIP-induced reduction in viability was 15 – 32% below the mean viability of the respective control. Both concentrations of VIP were equally active. Gastrin produced 17 – 30% increase in neutrophil viability in 7 subjects with both concentrations of the

hormone being equally active. However, in two volunteers the *lower* concentration of gastrin was suppressive to viability. GIP produced a suppressive effect in one volunteer. Secretin and CCK did not show detectable changes in neutrophil viability.

Three of the 7 subjects in whom gastrin enhanced viability were among the 8 subjects who showed no response to VIP i.e. there is partial overlap in responsiveness to VIP and gastrin.

Table 1: In vitro effects of VIP, Gastrin and GIP on neutrophil viability.

Subject Groups Responding	**VIP**	**Gastrin**	**GIP**
Number of Subjects with Increased Viability (*Magnitude of change*)	1 *19% increase*	7 *Median increase= 22% Range= 17 - 30%*	None
Number of Subjects with Decreased Viability (*Magnitude of change*)	11 *Median decrease = 27% Range= 15 - 32%*	2 *20% and 27% decrease*	1 *24%*

For each individual, the hormone-induced change in neutrophil viability is expressed as a percentage of the respective control. The median and the range of percent viability changes are shown for each group in italics.

viability for the 11 subjects responding to VIP treatment. The difference in viability between controls and tests was statistically significant with $P = 0.031$. The gastrin-induced increase in viability for the 7 subjects was statistically significant with $P = 0.044$.

Changes in caspase levels

Based on the results of the neutrophil viability tests shown in Table 1, a second sample of peripheral venous blood was taken from the 14 volunteers whose neutrophils were affected by gastrin and/or VIP. Caspase levels were measured at 1 hour, 2 hours, 4 hours and 6 hours of incubation. The largest differences in caspase levels between hormone-treated neutrophils and controls were at 2 and 4 hours of culture. The largest difference in caspase level for each subject is expressed as a percentage of the respective control and used for comparisons with others.

Table 2 shows that VIP induced an increase in the levels of caspase 3/7 and caspase 8 in most of the 11 subjects in whom VIP caused depression of neutrophil viability. The seven subjects with gastrin-induced increased neutrophil viability showed varying responses; one subject had increased levels of both caspase 3/7 and caspase 8, four subjects had decreased caspase 3/7 only and two subjects showed no changes at all. Neither VIP nor gastrin produced detectable changes in the levels of caspase 9.

Table 2: *In vitro changes of neutrophil caspase levels in subjects with VIP-induced and gastrin-induced modulation of neutrophil viability.*

Subject Groups Responding	**Caspase 3/7**		**Caspase 8**		**Caspase 9**	
	VIP N = 11	Gastrin N = 7	VIP N = 11	Gastrin N= 7	VIP N= 11	Gastrin N= 7
Number of Subjects with Increased Caspase Level (*Range*)	9 (*18 - 54%*)	1 (*10%*)	10 (*40 - 557%*)	1 (*31%*)	None	None
Number of Subjects with Decreased Caspase Level (*Range*)	None	4 (*11 - 28%*)	None	None	None	None

Statistical significance of hormone-induced viability changes

The paired t-test was used to test the significance of the VIP-induced reduction in neutrophil

Neutrophils tested for caspase levels are from the volunteers in whom there was VIP-induced reduction of viability and those in whom there

was gastrin-induced increase in viability (Table 1). "N" in each column is the number of samples treated with the hormone and then tested for the caspases. The hormone-induced change in the level of a caspase for each subject is presented as a percentage of the respective control.

Discussion

The neutrophil granulocytes are pillars of innate immunity and changes in their life span at sites of inflammation appear to be quite an important factor in determining the course of events. Mature neutrophils die rapidly by apoptosis, both *in vitro* and *in vivo*, unless they are rescued by environmental signals like the proinflammatory cytokines IL-2, IL-8 and IFN-γ which suppress apoptosis.[13]

This study aimed to investigate the effects of 5 gut hormones on the viability and spontaneous apoptosis of human neutrophils *in vitro*. The first stage of the investigation explored the effects of VIP, gastrin, GIP, CCK and secretin on neutrophil viability. It showed that VIP and gastrin were the only hormones most active in influencing neutrophil viability. Therefore, only VIP and gastrin were included in the second stage of the study which investigated the changes in initiator and executor caspase levels.

Our results suggest that VIP accelerated apoptosis of human neutrophils in many, but not all individuals and that gastrin inhibited apoptosis in some, but not all individuals. An individual could therefore be responsive or non-responsive to these gut hormones. We refrain from calculating the exact proportion of individuals responsive to these gut hormones because our sample size is small and because it is not representative of the whole population. We see our results as a stimulus for more detailed studies.

Our results also suggest that the effects of VIP and gastrin on the life span of neutrophils were not uniform with respect to the nature of the effect and the timing of the effect. VIP primarily reduced neutrophil viability, but it did increase it in one subject. Gastrin was mostly an enhancer of neutrophil viability, but it did reduce it in two subjects. The culture time at which the maximum effect of a hormone on neutrophil viability was detected, varied between 2 and 6 hours. As yet, we cannot explain this lack of uniformity nor can we explain why some individuals are not responsive at all. The increase in caspase 8 and caspase 3/7 produced by VIP is consistent with its ability to reduce neutrophil viability. In case of gastrin the caspase changes were less consistent.

It has been reported that VIP could reduce or prevent lung injury in animal models of the acute respiratory distress syndrome and other lung disorders associated with impaired cell viability.[3] The ability of VIP to modulate apoptosis was therefore tested in various experimental systems. A study using rat alveolar cells and a related cell line concluded that VIP improved cell viability by inhibiting apoptosis induced by tumour necrosis factor, Fas ligand and other agents.[3] Another study also reported that VIP inhibited activation induced lymphocytes death.[4] Unlike these two reports, apoptosis in our study was spontaneous, not induced. However, a report by Djanani and Kähler [2] concluded that VIP did not modulate apoptosis in human neutrophils. The different experimental systems could explain some of the discrepancies between our findings and those of the other researchers regarding the effects of VIP on apoptosis. However, we propose that these discrepancies could also be partially explained by our two observations that:

1- Not all individuals are responsive to VIP and gastrin, and
2- The same hormone could produce opposing effects in different individuals.

We recommend that these two points be considered by investigators before the final analysis is done and conclusions drawn about the effects of these peptides.

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